II. REMARKS

Formal Matters

Claims 1-3, 5-8, 20, 33, 63, and 64 are pending after entry of the amendments set forth herein.

Claims 1-8, 16, 20, 33, 63, and 64 were examined and were rejected. Claims 9-15, 17-19, 21-26, 28-32, and 34-62 were withdrawn from consideration.

Claims 1, 2, 20, 33, and 63 are amended. The amendments to claims 1, 2, 20, 33, and 63 were made solely in the interest of expediting prosecution, and are not to be construed as an acquiescence to any objection or rejection of any claim. Support for the amendments to claims 1, 2, 20, 33, and 63 is found in the claims as originally filed, and throughout the specification, in particular at the following locations: "neural progenitor cell": page 12, lines 20-25; and page 68, lines 4-10; "parenteral administration": page 34, lines 9-11; "to an individual having CNS damage or lesion": page 30, lines 13-21; and page 68, lines 4-10; "wherein said administering effects migration of the neural progenitor cell or progeny thereof to the site of damage or lesion": page 29, line 25 to page 30, line 21; page 59, lines 13-17; page 62, lines 3-5; and page 68, lines 4-10. Accordingly, no new matter is added by these amendments.

Claims 4, 9-19, 21-32, and 34-62 are canceled without prejudice to renewal, without intent to acquiesce to any rejection, and without intent to surrender any subject matter encompassed by the canceled claims. Applicants expressly reserve the right to pursue any canceled subject matter in one or more continuation and/or divisional applications.

Applicants respectfully request reconsideration of the application in view of the remarks made herein.

Examiner Interview

The undersigned Applicants' representative thanks Examiner Turner and Examiner Kunz for the courtesy of an in-person interview which took place on June 25, 2003, and which was attended by Examiners Turner and Kunz, inventor Dr. Jim Fallon, and Applicants' representatives Carol L. Francis and Paula A. Borden.

During the interview, the rejection of claims 1-8, 16, 20, and 23 under 35 U.S.C.§112, first paragraph, was discussed. The phrase "outside the ventricles" was discussed in connection with the rejection under 35 U.S.C.§112, first paragraph. During the interview, the rejection of claims 1-8, 16, 20,

and 23 under 35 U.S.C. 102(e) was discussed. The differences between the instant invention as claimed and the cited art were discussed.

Rejections under 35 U.S.C.§112, first paragraph

Claims 1-8, 16, 20, and 33 were rejected under 35 U.S.C.§112, first paragraph, as allegedly reciting new matter. Claims 1-8, 16, 20, and 33 were rejected under 35 U.S.C.§112, first paragraph, as allegedly lacking written description. Claims 1-8, 20 and 33 were rejected under 35 U.S.C.§112, first paragraph, as allegedly lacking enablement.

Claims 1-8, 16, 20, and 33; "new matter"

The Office Action stated that claims 1, 20, and 33 were amended to recite "wherein said administration is outside the ventricles," and stated that the specification does not support the limitation. As explained during the Examiner Interview, the specification discusses administration outside the ventricles. For example, the specification discusses experiments in which TGF-α was administered intrastriatally. Specification, page 49, lines 15-17; page 51, lines 5-6; and page 69, lines 1-6. Applicants note that intrastriatal administration is "outside the ventricles." Accordingly, the specification provides support for administration outside the ventricles. During the Examiner Interview, it was agreed that the specification supports the phrase "wherein said administration is outside the ventricles." Withdrawal of this rejection is respectfully requested.

Claims 1-8, 16, 20, and 33; written description

The Office Action stated that, while the disclosure teaches that TGF-α is a molecule that binds EGF receptor, the recitation of alternatively described molecules which merely maintain such functional activities represents a functional recitation of multiple distinct classes of molecules which are not structurally described but which are encompassed by the claims.

The Office Action stated that there appears to be no single embodiments disclosed for the recitation of the second compound in claim 27. Applicants note that claim 27 was previously canceled, and therefore this rejection is moot.

Applicants respectfully traverse the written description rejection of claims 1-8, 16, 20, and 33. Applicants' position with respect to claims 1-8, 16, 20, and 33 has been previously made of record.

Nevertheless, and solely in the interest of expediting prosecution, claim 1 is amended to recite administration of TGF-α or a functional fragment thereof.

Claims 1-8, 20, and 33; enablement

The Office Action stated that the specification does not reasonably provide enablement for the generic recitation of all compounds which bind to the EGF receptor to attract a glial progenitor cell or progeny thereof or to stimulate differentiation as claimed.

Applicants respectfully traverse the enablement rejection of claims 1-8, 20, and 33. Applicants' position with respect to claims 1-8, 20, and 33 has been previously made of record. Nevertheless, and solely in the interest of expediting prosecution, claim 1 is amended to recite administration of TGF-α or a functional fragment thereof. As discussed during the interview, the amino acid sequence and structure of TGF-α were well understood at the time of the invention, and thus the present claims pose no issues with respect to enablement under 35 U.S.C. §112, first paragraph.

Conclusion as to the rejections under 35 U.S.C.§112, first paragraph

Applicants submit that the rejection of the claims discussed above under 35 U.S.C. §112, first paragraph, has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

Overview of the Claimed Invention

Before addressing the rejection of the claims based on the art, applicants take this opportunity to provide an overview of the claimed invention.

The present claims are directed to:

1) parenteral administration of TGF-α polypeptide or functional fragment to an individual having CNS damage or lesion, where the TGF-α administered outside the ventricles so as to effect migration of neural progenitor cells (or progeny thereof) to the site of CNS damage or lesion (independent claim 1);
2) administration of TGF-α polypeptide or functional fragment to a site of CNS damage or lesion in an individual, where the TGF-α administered outside the ventricles, so as to attract neural progenitor cells (or progeny thereof) to the site of CNS damage or lesion (independent claim 33); and

3) administration of TGF-α polypeptide or functional fragment to a site of CNS damage or lesion in an individual, where the TGF-α administered intrastriatially (*i.e.*, at a site outside the ventricles), so as to attract neural progenitor cells (or progeny thereof) to the site of CNS damage or lesion (independent claim 33).

As discussed by Dr. Fallon during the interview, it is the combination of the injury signal and TGF-α administration outside the ventricles (or, more specifically, intrastriatally or at the site of injury) that results in therapeutically significant proliferation and directed migration of progenitor cells (or their progeny) toward the site of injury.

Administration of TGF-α inside the ventricles as taught by Weiss simply does not provide this combination. It is neither taught nor suggested by the cited art (see rejection based on Weiss below). Instead, Weiss teaches, and particularly in the context of treatment that involves CNS damage or lesion, growth factors are to be administered into the ventricles (see, e.g., col. 26, lines 15-26). Weiss defines "ventricles" as "any cavity or passageway within the CNS through which cerebral spinal fluid flows" (see col. 13, lines 4-11).

We now turn to the outstanding rejections as set forth in the Office Action.

Rejection under 35 U.S.C.§102(e)

Claims 1-8, 16, 20, 33, 63, and 64 were rejected under 35 U.S.C.§102(e) as allegedly anticipated by U.S. Patent No. 5,980,885 ("Weiss").

The Office Action stated that Weiss teaches administration of TGF-α to brain for the purpose of inducing *in vivo* proliferation, migration, and differentiation of neural and/or glial cells and for treatment of Huntington's, Alzheimer's, Parkinson's and other neurological disorders. The Office Action stated that since the treatment of Weiss involves administration of TGF-α to the brain and results in the contact of TGF-α with neural progenitor cells within the brain, the treatment taught by Weiss is necessarily the same as that claimed, because the treatment comprises the same reagents, steps, and effects noted. Applicants respectfully traverse the rejection.

As discussed during the Examiner Interview, Weiss does not disclose or suggest the instant methods as claimed.

During the Examiner Interview, Dr. Fallon explained that administration of a growth factor such as TGF-α into the ventricles as taught by Weiss is not effective to stimulate migration of a neural progenitor cell or progeny thereof to a site of damage or lesion in the CNS. Dr. Fallon further explained that administration of a growth factor such as TGF-α alone, in the absence of CNS damage or lesion, is ineffective to stimulate migration of a neural progenitor cell or progeny thereof. Instead, both administration of a growth factor such as TGF-α and the presence of CNS damage or lesion are required to stimulate migration of a neural progenitor cell or progeny thereof to a site of damage or lesion in the CNS.

As Dr. Fallon noted during the Examiner Interview, the fact that CNS damage or lesion must be present in order to stimulate migration of a neural progenitor cell or progeny by administering a growth factor such as TGF-α is discussed in Fallon et al. ((2000) *Proc. Natl. Acad. Sci. USA* 97:14686-14691; a copy of which is provided herewith as Exhibit 1.)

Weiss neither discloses nor suggests administration of a growth factor such as TGF-α outside the ventricles to an individual having CNS damage or lesion.

The Examiner pointed to general disclosure in Weiss of routes of administration of growth factors (see col. 25, lines 41-60). However, Applicants submit that Weiss' entire focus is upon administration of growth factors to the ventricles, which is apparent upon a close reading of, for example, col. 25, line 41 to col. 26, line 64. Weiss repeatedly states that growth factors are administered to the ventricles or to other spaces continuous with the ventricles (*e.g.*, administration to the lumbar cistern for "circulation throughout the CNS", col. 26, lines 63-64).

Of most importance here, when discussing administration of a growth factor in the context of treating a CNS disorder in an individual having a CNS damage or lesion, Weiss states that a growth factor is administered **to the ventricles** (see, *e.g.*, Weiss, column 26, lines 15-26). This is exactly the opposite of what is claimed. Thus, when discussing administration in the context of stimulating migration of a neural progenitor cell or progeny thereof to a site of CNS damage or lesion, Weiss repeatedly instructs administration into the ventricles. Weiss' definition of "ventricle", as noted above,

makes it clear that this is the opposite of the claimed invention, which requires administration **outside** the ventricles.

Examples 27-30 of Weiss discuss administration of EGF, FGF, or a combination of EGF and FGF to the ventricles. Again, this is opposite of what is claimed.

In the context of treating Huntington's Disease, Parkinson's Disease, and Alzheimer's Disease, Weiss states that "growth factors or other neurological agents would be delivered to the ventricles of the forebrain to affect in vivo modification or manipulation of the stem cells." Weiss, column 26, lines 16-21, emphasis added. In the context of treating Parkinson's Disease, Weiss states that "Dopamine cells can be generated in the striatum by the administration of a composition comprising growth factors to the lateral ventricle." Weiss, column 26, lines 38-43, emphasis added. Once again, this is opposite of what is claimed.

Weiss discusses genetic modification of neural precursor cell *in vitro* so that the cell secretes a growth factor. Weiss, column 21, lines 1-9. Weiss discusses implantation of genetically modified precursor cells into the CNS of a recipient. Weiss also discusses transplantation of non-transfected cells in a Huntington's Disease animal model, and a Parkinson's Disease animal model. Weiss, column 60, lines 12-67. However, the instant methods as claimed recite administration of a polypeptide, not a genetically modified precursor cell. Administration of a cell is not equivalent to administration of a polypeptide. Thus, this aspect of the disclosure of Weiss is not relevant to the instant claims.

As also discussed during the interview, Dr. Fallon explained that TGF-α administration into the ventricles simply does not provide for a therapeutically significant proliferation of progenitor cells, and no detectable migration toward the site of injury. As Dr. Fallon explained, there are a number of barriers between the ventricles and the brain proper. Stem cells resident within the ventricles normally migrate through the ventricles toward the olfactory bulb, and do not migrate through these barriers into the brain proper.

Weiss itself, particularly in Examples 27-30, evidence the effect of this barrier. Intraventricular administration of a growth factor to non-injured animals resulted in meager, background level, and certainly therapeutically insignificant, proliferation (an increase of from about 20 to 350 cells) with non-

directed migration (Column 27, lines 17-26). As explained by Dr. Fallon during the interview, the "increase" observed by Weiss is on the order of "background noise" in contrast to the therapeutically relevant increase provided by the claimed invention. Moreover, as evidenced in the instant specification, administration of TGF-α inside the ventricles (i.e., by intracerebroventricular infusion) does not induce migration (as detected by formation of a striatal ridge). Specifically:

> All rats receiving infusions into brain areas other than the striatum received two-week TGFα infusions and nigral 6-OHDA lesions. Intracerebroventricular (ICV) infusion of growth factor ipsilateral to the lesion stimulated the buildup of cells in the adjacent ventricular wall, but did not induce formation of the striatal ridge in any of the animals.

(specification page 57, lines 7-11; emphasis added)

As discussed above, Weiss neither discloses nor suggests a method of attracting a neural progenitor cell, or a progeny of a glial progenitor cell, to a site of CNS damage or lesion, the method comprising administering a growth factor such as TGF-α to an individual having a CNS damage or lesion, wherein administration is outside the ventricles, wherein the neural progenitor cell or progeny thereof migrates to the site of damage or lesion in the CNS. Accordingly, Weiss cannot anticipate the instant invention as claimed.

Applicants submit that the rejection of claims 1-8, 16, 20, 33, 63, and 64 under 35 U.S.C. §102(e) has been adequately addressed in view of the remarks set forth above. The Examiner is thus respectfully requested to withdraw the rejection.

III. CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided. Should the undersigned not be available, the Examiner is invited to telephone Carol L. Francis at the number provided.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number UCAL263CIP.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: July 22, 2003

By: _____
Paula A. Borden
Registration No. 42,344

BOZICEVIC, FIELD & FRANCIS LLP
200 Middlefield Road, Suite 200
Menlo Park, CA 94025
Telephone: (650) 327-3400
Facsimile: (650) 327-3231

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In vivo induction of massive proliferation, directed migration, and differentiation of neural cells in the adult mammalian brain

James Fallon*†, Steve Reid*, Richard Kinyamu*, Isaac Opole*, Rebecca Opole*, Janie Baratta*, Murray Korc‡, Tiffany L. Endo*, Alexander Duong*, Gemi Nguyen*, Masoud Karkehabadhi*, Daniel Twardzik§, and Sandra Loughlin¶

Departments of *Anatomy and Neurobiology, ‡Medicine, and ¶Pharmacology, University of California, Irvine, CA 92697-1275; and §Stem Cell Pharmaceuticals, Seattle, WA 98104

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The development of an *in vivo* procedure for the induction of massive proliferation, directed migration, and neurodifferentiation (PMD) in the damaged adult central nervous system would hold promise for the treatment of human neurodegenerative disorders such as Parkinson's disease. We investigated the *in vivo* induction of PMD in the forebrain of the adult rat by using a combination of 6-hydroxydopamine lesion of the substantia nigra dopaminergic neurons and infusions of transforming growth factor α (TGFα) into forebrain structures. Only in animals with both lesion and infusion of TGFα was there a rapid proliferation of forebrain stem cells followed by a timed migration of a ridge of neuronal and glial progenitors directed toward the region of the TGFα infusion site. Subsequently, increasing numbers of differentiated neurons were observed in the striatum. In behavioral experiments, there was a significant reduction of apomorphine-induced rotations in animals receiving the TGFα infusions. These results show that the brain contains stem cells capable of PMD in response to an exogenously administered growth factor. This finding has significant implications with respect to the development of treatments for both acute neural trauma and neurodegenerative diseases.

Neurogenesis in the adult mammalian central nervous system has been demonstrated in the dentate gyrus of the hippocampus and in the olfactory bulbs of adult birds and rodents, and it has been shown that these cells arise from a rostral migratory stream originating in the subventricular zone (SVZ) (1–3). The SVZ is rich in pluripotent stem cells (4–7), and such cells have also been demonstrated to exist in varying degrees throughout the neuraxis (8–10). While it has been shown that these cells can proliferate *in vitro* in response to extracellular signals and growth factors such as fibroblast growth factor, basic fibroblast growth factor, and ligands that bind the type I family of tyrosine kinase receptors, including epidermal growth factor (EGF) and transforming growth factor α (TGFα) (11–13), *in vivo* studies of proliferation, migration, and differentiation (PMD) have met with only limited success (14, 15). Successful demonstration of *in vivo* PMD in neural tissue would provide tools to begin to develop drugs to treat neurodegenerative diseases such as Parkinson's disease.

Recent evidence suggests that a combination of extracellular signals and microenvironmental conditions may be necessary for the *in vivo* stimulation of neuroepithelial stem cells, including extracellular matrix molecules, paracrine and juxtacrine cell–cell signaling, and growth factor delivery and concentration (16). Magavi *et al.* (17) have demonstrated local *in vivo* neurogenesis in adult mice in response to microenvironmental modification by targeted apoptosis. However, to date, no studies have demonstrated the induction of the massive proliferation, directed migration, and differentiation of endogenous neuroepithelial stem cells into neurons in the broader regions of the nervous system, which would allow repopulation of, and regeneration of, damaged neural tissue. In the present study, we demonstrate the *in vivo* induction of massive PMD in the forebrain of the adult rat following unilateral 6-hydroxydopamine (6-OHDA) lesioning of the substantia nigra dopaminergic neurons and infusions of TGFα into ipsilateral forebrain structures.

Materials and Methods

Adult male Sprague–Dawley albino rats (240–350 g) (n = 130) were obtained from Simonsen Laboratories (Gilroy, CA). The animals were maintained in a temperature- and humidity-controlled campus vivarium. All aspects of animal handling and surgery were in accordance with current National Institutes of Health guidelines and University of California at Irvine Institutional Animal Care and Use Committee protocols. 6-OHDA and TGFα were administered as follows.

6-OHDA Lesions. Animals were anesthetized with i.p. xylazine (8 $mg \cdot kg^{-1}$) and ketamine (100 $mg \cdot kg^{-1}$) (Western Medical Supply, Arcadia, CA). A chilled sterile solution of 1.0–4.8 mg/ml of 6-OHDA (Sigma) in 0.9% saline with 0.01% ascorbic acid was prepared immediately before injection. The animals were immobilized in a Kopf stereotaxic device and placed on a warm mat at 37°C. Using aseptic techniques, 2.5–8 μl of 6-OHDA solution was stereotaxically injected at the rostral border of the substantia nigra–ventral tegmental area (SN-VTA) (+3.7 A/P; +2.1 M/L; +2.0 D/V) at a rate of 1 $\mu l \cdot min^{-1}$ using interaural zero as a reference. The total duration of surgery was approximately 45 min.

Growth Factor Infusions. Osmotic minipumps (models 2002 and 2004, Alzet) were used for TGFα and artificial cerebrospinal fluid (aCSF) infusions. They were implanted at a predetermined period contemporaneous with, or after, the 6-OHDA lesion. The minipumps were filled with approximately 200 μl of a solution containing 10, 50, or 100 μg of TGFα (Stem Cell Pharmaceuticals) in aCSF for experimental animals or plain aCSF for control animals and incubated overnight in normal saline at 37°C before implantation. Under aseptic conditions, the 5-mm cannula attached to the minipump (Brain Infusion Kit, Alzet) was stereotaxically implanted into the left caudate–putamen (+1.2

Abbreviations: PMD, massive proliferation, directed migration, and neurodifferentiation; TGFα, transforming growth factor α; SVZ, subventricular zone; EGF, epidermal growth factor; EGFr, epidermal growth factor receptor family; 6-OHDA, 6-hydroxydopamine; aCSF, artificial cerebrospinal fluid; TH, tyrosine hydroxylase; SN-VTA, substantia nigra–ventral tegmental area; DAT, dopamine transporter.

†To whom reprint requests should be addressed. E-mail: jfallon@uci.edu.

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A/P; +2.7 M/L) using bregma as a reference. The minipump was placed s.c. in the interscapular region. The infusate was delivered directly into the striatum by the minipump at a rate of approximately 0.5 $\mu l \cdot h^{-1}$. The animals (n = 130) were subdivided into groups receiving simultaneous infusions (TGFα, n = 25; aCSF, n = 13), infusions 2 weeks after 6-OHDA (TGFα, n = 20; aCSF, n = 11), controls receiving infusions without a 6-OHDA lesion (TGFα, n = 8; aCSF, n = 8), intraventricular infusion with 6-OHDA lesion (n = 2), or 6-OHDA lesion without any infusion (n = 43).

Behavior Testing. The apomorphine-induced rotation technique was used to test behavioral changes in both the 6-OHDA lesioned and nonlesioned animals. A fresh solution of apomorphine (0.25 mg/ml) was prepared each time. The animal was set on a rotometer bowl and its behavior was observed for 5 min. It then received an s.c. injection of apomorphine (2.5 mg/kg body weight) and was placed back in its cage and allowed to rest for 10 min. Apomorphine-induced rotations were thereafter observed at 10-min intervals, each session lasting 5 min for a total of 15 min.

***In Situ* Hybridization Histochemistry.** TGFα mRNA probes were generated from a 550-nt *Xba*I–*Bam*HI cDNA fragment from the 5′ end of rat TGFα, subcloned into pGEM 7Zf (Promega). Antisense and sense probes were transcribed with SP6 and T7 polymerases, respectively. Rat EGF receptor (EGFr) mRNA probes were produced from a 718-bp *Bam*HI–*Sph*I insert from the 5′ end of the gene in pGEM 7Zf. Probes for rat tyrosine hydroxylase (TH) were created by using the 1.3-kDa *Bam*HI–*Eco*RI fragment subcloned into pGEM 7Zf. Antisense subclones for EGF receptor and TH were transcribed with T7 polymerase. Sense subclones for EGF receptor and TH were transcribed with SP6 polymerase. All probes were radiolabeled by transcription in the presence of ^{35}S-labeled UTP (NEN).

In situ nucleic acid hybridization was performed according to Simmons *et al.* (44). Parallel sections from experimental and control animals were hybridized overnight at 65°C with sense or antisense probes at a concentration of 107 cpm/ml. Adjacent sections from the same animals were hybridized to each of the probes so that direct comparison could be made of their anatomical distributions.

Slides from experimental and control animals were grouped together and apposed with ^{14}C-labeled brain paste standards to autoradiographic Beta Max Hyperfilm (Amersham Pharmacia) for 3–7 days. After successful development of the autoradiography film, analysis and quantitation was done by using MCID (Imaging Research, St. Catherine's, ON, Canada). Densitometry readings were sampled at multiple sites within each anatomical region of interest and averaged. Relative concentrations of TGFα and EGF receptor from the hybridization process were then estimated by using a computer-generated third-degree polynomial standard curve constructed from the ^{14}C brain paste standards. The estimated values for each region in each treatment group were then averaged and their standard errors were calculated. Brain regions ipsilateral to the experimental treatments were compared with the corresponding regions in control brains at approximately the same positions. Significance of the comparisons was determined by using the Student t test.

BrdUrd Administration. Animals received 50 mg/kg BrdUrd (Boehringer Mannheim) i.p. hourly for 3 days from the day of surgery.

Tissue Preparation. Animals were killed at timed intervals between 1 and 28 days by either decapitation or perfusion. After decapitation, the brains were quickly removed and frozen in isopentane at −20°C. Coronal cryostat sections were cut at 40 μm and thaw adhered to Vectabond (Vector Laboratories)-coated slides. The sections were postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, rinsed in phosphate buffer and air dried, then stored at −20°C until processed.

After cardiac perfusion first with saline then 4% paraformaldehyde, the brains were quickly removed and postfixed in 4% paraformaldehyde for 1 h, then cryoprotected in 30% sucrose overnight at 4°C. The brains were then cooled and cut at −20°C on a freezing microtome.

Immunohistochemistry. Perfused brain tissue was cut on a freezing microtome at 40 μm, and free-floating sections were placed in 0.1 M PBS, pH 7.4. Blocking was done by using 10% normal goat serum with 0.4% Triton X in PBS for 1 h before primary antibody incubation. Primary antibodies were dissolved in blocking solution as follows. Rabbit anti-glial fibrillary acidic protein (1:400, Dako), mouse anti-β-III tubulin (1:100, R & D Systems), rabbit anti-β-III tubulin (1:10,000, R & D Systems), rabbit anti-tyrosine hydroxylase (1:1,000, Sigma), rabbit anti-s100β (1:2,500, R & D Systems), mouse anti-nestin (1:20, Hybridoma Bank), mouse anti-doublecortin (1:250, kind gift of C. Walsh), and rat anti-dopamine transporter (DAT; Chemicon, 1:5,000). The tissues were then incubated overnight at room temperature in primary antibody and then rinsed in PBS and incubated in biotinylated or fluorescent secondary antibodies (anti-mouse, anti-rabbit, anti-rat) in blocking solution. Biotinylated primaries were visualized by using ABC solution (Vector ABC Elite) for 1 h, followed by diaminobenzidine-peroxidase histochemistry (Sigma).

For BrdUrd immunocytochemistry, DNA was denatured by using 50% formamide in 2× SSC for 2 h at 65°C, and tissue sections were then rinsed in 2× SSC for 5 min and incubated in 2 M HCl at 37°C for 30 min. The tissue slices were then rinsed in 0.1 M boric acid, pH 8.5, for 10 min, followed by a 5-min rinse in PBS. For double labeling, tissues were incubated with anti-BrdUrd and either anti-TH or anti-DAT primary antibodies, then processed for fluorescence as above.

Silver Staining for Cellular Morphology. Cells were labeled by using a modification to the Nauta method, similar to procedure 1 of Fink-Heimer (18). Free-floating sections were placed into 0.05% potassium permanganate before treatment with fresh 1% hydroquinone/1% oxalic acid and were then treated with successive uranyl nitrate/silver nitrate solutions of increasing concentration. After another rinse, the sections were reacted in ammoniacal silver, then in ethanol/citric acid/paraformaldehyde reducer, and finally in sodium thiosulfate. After staining, sections were mounted on glass slides, dehydrated, and cover-slipped.

Results

Morphological Studies of PMD Induction. We first determined conditions sufficient to induce the massive proliferation of multipotential stem cells originating in the SVZ and the subsequent directed migration of these neuroprogenitor cells into the striatum. We used combinations of infusions of TGFα into the ipsilateral striatum, and 6-OHDA injections into the ipsilateral SN-VTA (Fig. 1). Because TGFα-responsive stem and neuroprogenitor cells in the SVZ have been shown to express EGFr mRNA, we used *in situ* nucleic acid hybridization and other anatomical and behavioral techniques to demonstrate TGFα responsiveness in the SVZ. In animals receiving 6-OHDA lesions of SN-VTA and aCSF control infusions into the striatum, there was no significant change in the expression of EGFr mRNA in the SVZ (Fig. 1*a*). In a second control group receiving TGFα infusions, but no 6-OHDA lesions, there was a significant increase in the expression of EGFr mRNA in the SVZ for the duration of the infusion (Fig. 1*b*). In the experimental group

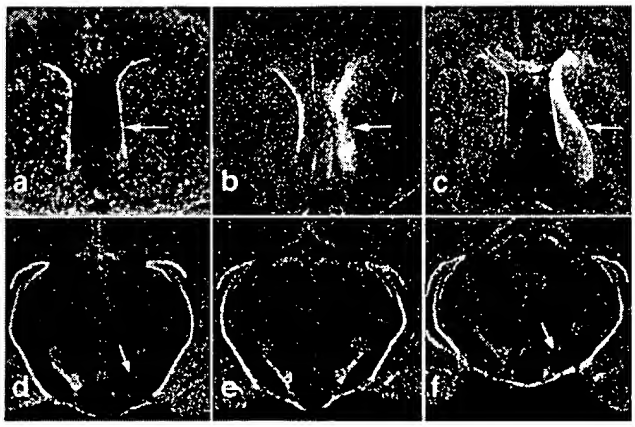

Fig. 1. Autoradiograms of coronal sections of forebrain (*a–c*) and midbrain (*d* and *e*) showing EGFr mRNA expression in the SVZ (arrows in *a–c*) and SN-VTA (*d–f*), respectively. Animals received 6-OHDA lesion of SN-VTA and aCSF infusion into striatum of right side of brain (*a* and *d*), TGFα infusion into striatum but no lesion of SN-VTA (*b* and *e*), or both a 6-OHDA lesion of SN-VTA and a TGFα infusion into striatum (*c* and *f*). There is a significantly higher (200%, $P < 0.01$) expression of EGFr mRNA in the SVZ of animals receiving TGFα (*b* and *c*). When both a 6-OHDA lesion and TGFα infusion are made, an EGFr-mRNA positive ridge lateral to the SVZ is present in the striatum (*c*). The density of EGFr-mRNA in nonridge areas of striatum was unchanged after TGFα infusions ($P < 0.001$). Arrows in *d* and *f* point to the right substantia nigra. The lack of an EGFr *in situ* signal at these sites confirms that the 6-OHDA injection on the right side lesioned the majority of EGFr-expressing dopaminergic neurons in this region.

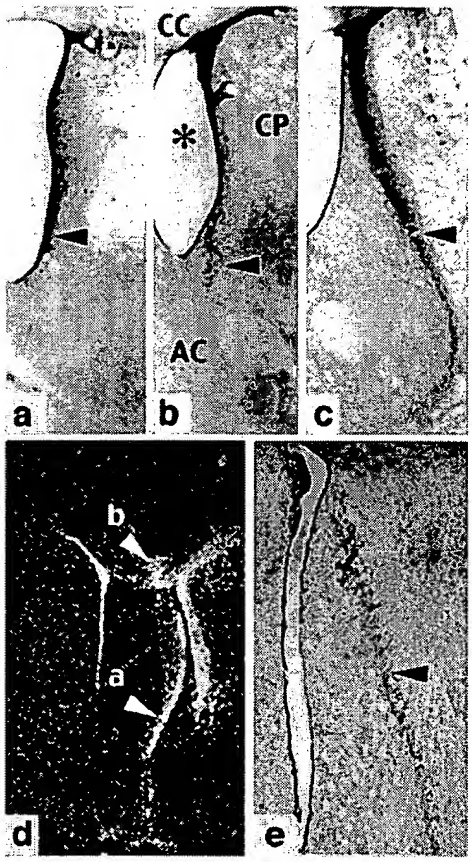

Fig. 2. Time-course analysis of SVZ proliferation and progression of ridge migration. There is an initial proliferation of SVZ cells (arrowhead in *a*) in the first week of TGFα infusion into the caudate-putamen (CP) (*a*), followed by an en masse migration of the ridge into the striatum (arrowhead), starting at 9 days of TGFα infusion (*b*), progressing to the midstriatum by the 14th day (AC, anterior commissure) (*c*) *, Lateral ventricle. Septal infusions resulted in a medially directed EGFr-mRNA positive "septal" ridge (*d*, arrowhead a) originating in the medial SVZ adjacent to the striatum (*d*). Several other migration patterns can be seen, for example a colossal ridge (*d*, arrowhead b) or in the septum of the contralateral medial SVZ after striatal TGFα infusions (*e*).

receiving both a 6-OHDA lesion and a TGFα infusion, there was also a significant increase of EGFr mRNA expression in the SVZ. In animals that received more than 9 days of TGFα infusion, there was also an additional ridge (or thick-layered sheet in three dimensions) of EGFr mRNA-positive cells encroaching into the striatum from the SVZ (Fig. 1*c*). This ridge was most pronounced in animals that received concurrent 6-OHDA lesion and 10, 50, or 100 μg of total TGFα infusion, but also was present in animals receiving TGFα infusions starting 14 days after the 6-OHDA lesion. Both the 50- and 100-μg dose appeared to be equally effective, and the 10-μg dose was minimally less effective in inducing ridge formation.

In a serial time-course analysis, after 4–6 days (Fig. 2*a*) of continuous TGFα infusion in lesioned animals, there was a pronounced thickening of the SVZ. By 9 days (Fig. 2*b*), however, the ridge separates en masse from the SVZ, as well as at later time periods from 14 to 21 days, appearing progressively lateral to the SVZ toward the site of the TGFα infusion (Fig. 2*c*). The exact shape and apparent movement of the ridge depends on the site of the TGFα infusion. For example, a ventral striatal infusion results in an S-shaped ridge (Fig. 2*c*), whereas a dorsal striatal infusion resulted in a shorter and thicker ridge mass adjacent to the dorsal SVZ. The ridge of cells is observed whether the TGFα infusion is started concurrent with the lesion 2 days before or 14 days after the lesion. To determine whether this directed migration is limited to striatal targets, infusions of TGFα were made into the septum. In these cases, there was a massive proliferation of the SVZ, and a subsequent ridge appeared medially in the septum toward the TGFα infusion site (Fig. 2*d*). Additionally, in some cases a smaller ridge of cells could also be seen on the medial side of the contralateral SVZ in the septum (Fig. 2*e*) or migrating through the corpus callosum (Fig. 2*d*). In the cases where there was an infusion of TGFα into the lateral ventricle in 6-OHDA-lesioned animals, there was a temporary proliferation of SVZ cells but no migratory ridge.

Characterization of the Ridge. To further characterize the cells in the ridge, we used a series of morphological and immunocytochemical techniques (Fig. 3). Silver staining revealed fusiform cells in the ridge, oriented orthogonal to the most adjacent area of the SVZ. This is consistent with the possibility that these cells are migrating from the SVZ in parallel (Fig. 3*a*). After TGFα infusion into the brain, systemically administered 5′BrdUrd was incorporated into the SVZ with a specific and massively increased incorporation in the first 3 days. Subsequently, 5′-BrdUrd positive cells were seen in the ridge, striatum, external capsule, and cortex adjacent to the infusion cannula (Figs. 3*b* and 4*a*), indicating that the ridge cells were recently generated de *novo*. To determine the lineage and differentiation of the cells in and around the ridge, we first stained for nestin, a cytoskeletal marker for early lineage neuronal and glial progenitors. The SVZ and ridge cells were nestin-positive from the fourth day of TGFα infusion (Fig. 3*c*). To determine whether the ridge cells are of restricted glial and/or neuronal lineages, we stained for s100β (astrocytic lineage) and β-III tubulin (neuronal lineage). At 1, 4, 7, and 9 days (data not shown) of TGFα infusion, there was no evidence of significant positive staining of these markers, but starting at 14 days, β-III tubulin positive cells were seen (Figs. 3*d* and 4*b*). s100β positive cells were also seen in and

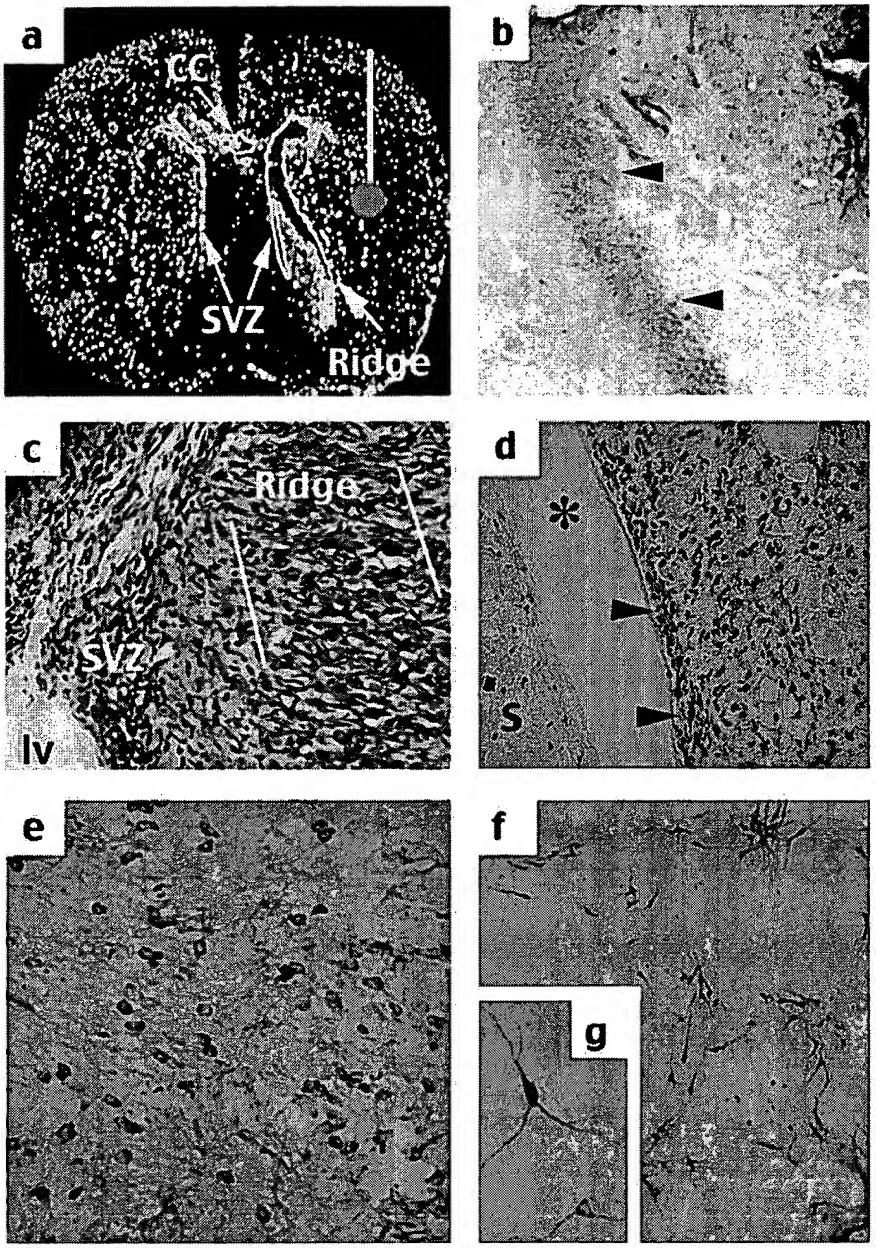

Fig. 3. Further characterization of the TGFα-induced striatal ridge cells. (*a*) Cross section of pseudocolor autoradiographic image (EGFr-mRNA) showing SVZ, location of a ridge, a smaller ridge in the corpus callosum (CC), and a cartoon image of the TGFα infusion cannula (white line) and infusion site in the right caudate putamen (pink circle). (*b*) The ridge cells (arrows) are nestin-positive, showing that they are neural progenitors. (*c*) Silver staining shows a fusiform morphology of the cells in the ridge (outlined by white lines), suggestive of outward migration from the SVZ lining the lateral ventricle (lv). (*d*) BrdUrd was incorporated by SVZ (arrows) and ridge cells laterally in the striatum, but not in the septum (S) after a striatal TGFα infusion. (*e*) Some migrating cells subsequently stained positive for β-III tubulin, a marker for neuronal restricted lineage. (*f*) Longer TGFα infusion times revealed increasing numbers of TH-positive neurons (higher magnification in *g*).

around the ridge (data not shown). Immunofluorescence staining was also carried out on adjacent tissue sections to verify that the ridge is densely populated with BrdUrd-positive cells (Fig. 4*a*) and that large clusters of β-III tubulin-positive neuronal precursors are present in the BrdUrd-rich ridge (Fig. 4 *b* and *c*). Doublecortin, a marker for migrating young neurons, is also present in the ridge cells (Fig. 4 *d* and *e*).

Staining for these restricted astrocytic and neuronal lineage markers was, however, greatly reduced by 28 days. To determine whether TGFα infusions in these same 6-OHDA-lesioned animals result in the new appearance of differentiated neurons in the striatum, TH and DAT immunoreactivity was carried out. Within the second to third week of infusion, occasional TH- and DAT-positive cells were seen near the infusion cannula. From the third and fourth week of infusion, increasing numbers of TH-positive (Fig. 3 *f* and *g*) and DAT-positive (Fig. 4 *f* and *h*) cells were seen in the striatum. In double-labeling experiments, some newly generated (BrdUrd-positive) neurons were also DAT-positive (Fig. 4 *f–h*) or TH-positive (data not shown).

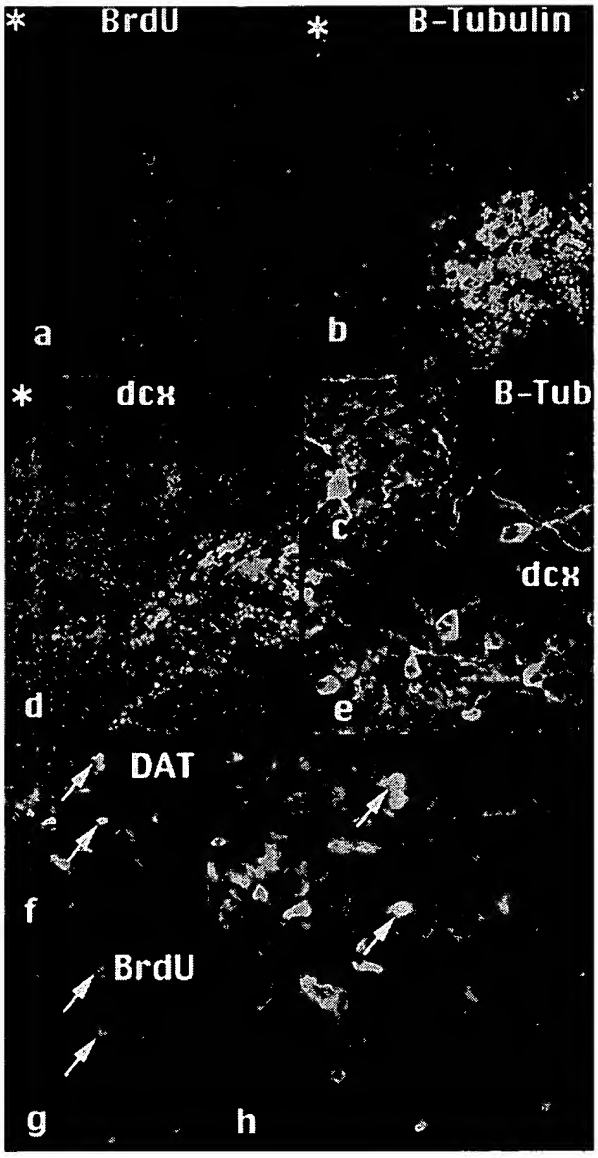

Fig. 4. Further characterization of migratory ridge cells by using fluorescence immunohistochemistry for neuronal markers. There is dense positive staining for BrdUrd (*a*), β-tubulin (*b* and *c*), and doublecortin (*d* and e). Labeling of the same section for DAT (*f*), and BrdUrd (*g*) reveals that some neurons are double-labeled for both markers (*h*). White arrows on *f–h* point to double-labeled neurons. *, Lateral ventricle.

Recovery of Function. To determine whether there is a functional correlate of the repopulation of neural cells in the striatum observed in the morphological and immunocytochemical studies, we carried out two sets of behavioral tests in some of these animals (Table 1). One group received a unilateral 6-OHDA lesion of the SN-VTA, and 14 days later, they received a 14-day continuous infusion of either aCSF or TGFα into the ipsilateral striatum. Rotational behavior in response to systemically administered apomorphine was tested before the lesion and then subsequently weekly for 4 weeks, both before and after the TGFα/aCSF infusions. In this rodent model of Parkinson's disease, there was a statistically significant ($P < 0.05$, two-tailed *t* test) 31.5% improvement in the rotational behavior of the TGFα versus aCSF-treated control animals.

A separate group of animals (individual animal data not shown) received TGFα, aCSF, or no infusions starting contemporaneously with the 6-OHDA lesion, as opposed to 2 weeks after the 6-OHDA lesion. In the groups that received either no infusion or an aCSF infusion, contralateral rotational behavior in response to an apomorphine injection was observed at each of 3- to 4-week intervals after the day when the animals received both the 6-OHDA injection and either aCSF infusion or no striatal infusion at all. In contrast, 10 of the 11 TGFα-treated animals did not exhibit rotational behavioral asymmetry following apomorphine injections during the entire 4-week period. Other motor behaviors such as exploratory behavior in the cage and consummatory behavior, on the other hand, did not appear to be adversely affected in the TGFα-treated animals.

Table 1. Behavioral results in Parkinson's disease model

Animal	Rotations/5 min Preinfusion	Postinfusion	% change
TGFα			
1	66.5	14.5	−78.2
3	48.0	14.5	−69.8
4	60.0	25.0	−58.3
5	60.0	40.0	−33.3
6	58.5	39.5	−32.5
7	163.5	140.5	−14.1
8	50.0	49.0	−2.0
9	33.0	33.0	0.0
11	103.5	185.7	79.4
Mean	71.4	60.2	−15.8
SD	37.2	57.1	
aCSF			
1	66.0	45.0	−31.8
2	158.5	151.0	−4.7
3	42.0	41.0	−2.4
4	40.0	40.0	0.0
5	71.0	71.0	0.0
6	94.0	96.0	2.1
7	38.0	41.0	7.9
8	144.5	158.5	9.7
9	63.5	100.5	58.3
10	101.0	162.0	60.4
11	84.0	138.0	64.3
Mean	82.0	94.9	15.7
SD	40.4	50.5	
t test	(2-tailed)		0.046

Apomorphine-induced rotations in 6-OHDA-lesioned animals, and 14 days later infused for 14 days with aCSF (control) or TGFα (experimental group). The order of animals is by descending order of % change of rotation. The percent improvement of rotational behavior (three 5-min periods) between the TGFα-treated animals and aCSF-treated animals was 31.5% (significant at $P < 0.05$, two-tailed *t* test). The TGFα animals received either 50 or 100 μg TGFα. Animals receiving 10 μg TGFα did not show significant improvement and were not included in this analysis.

Discussion

These findings provide evidence of induced coordinated proliferation, directed migration en masse, and phenotypic differentiation into TH-positive neurons and DAT (therefore, likely dopaminergic) of neural stem cells and their progenitors in the mammalian central nervous system *in vivo*. The parallel morphological and behavioral rotation experiments, coupled with the finding of newly generated (BrdUrd positive) and phenotypically specialized (TH-, DAT-positive dopamine neurons), indicate the usefulness of exogenous TGFα administration in the amelioration and reversal of symptoms of Parkinson's disease and other neurodegenerative disorders, as well as acute central

nervous system injury, for example, due to trauma and stroke. We found that the combination of TGFα infusion and endogenous stimuli arising from the injury signals in experimental animals resulted in a massive proliferation of the cells of the SVZ, followed by a directed migration en masse toward TGFα infusion sites in the striatum, septum, and external capsule or cortex. Further, we demonstrated a progressive pattern of proliferation, migration, maturation, and differentiation of newly generated cells, leading to neuronal and glial phenotypes with "spontaneous" differentiation and phenotypic specialization of some neurons, correlated with a desirable functional result (i.e., reversal of motor dysfunction).

The embryonic germinal layer of the central nervous system that is retained in the adult SVZ throughout the neuraxis is responsive to microenvironmental signals and can proliferate and differentiate in response to TGFα and other growth factors (19). Developmental changes in expression of the type I family of tyrosine kinase receptor family in neuroprogenitor cells have been shown to influence their proliferation, migration, and differentiation (16, 20). Both EGF and TGFα, members of the EGF family that bind to the other members of this family (Erb1 and possibly others) (21, 22), are present in the basal ganglia (23–26), although TGFα mRNA expression has been shown to greatly exceed that of EGF (27). TGFα has been shown to influence proliferation and migration of ganglionic eminence cells in the embryo (28) and proliferation of SVZ cells *in vivo* (29) and *in vitro* (30), a mechanism mediated via the EGF receptor (12, 31). The EGFr family of receptors is expressed in striatal and SVZ cells in both adult and developing brains (26, 32, 33). The observed increased expression of EGFr mRNA in the early stages of TGFα infusion in this study is consistent with a TGFα-dependent, EGFr-mediated mechanism of progenitor cell proliferation, as has been shown in other studies (31, 34).

Although progenitor cell proliferation in response to growth factors has been demonstrated *in vitro*, it has become clear that *in vivo* differentiation and migration requires additional microenvironmental signals. *In vitro* cell proliferation and migration and differentiation has been shown in response to microenvironmental cues provided by the substrate (e.g., fibronectin and/or integrins) (35) or altered in relation to neighboring cells, implying a cell–cell paracrine or contact mechanism (36). *In vivo*, however, it appears that microenvironmental manipulation using injury paradigms may also be effective in stimulating proliferation and migration of neural precursors, as demonstrated using several injury models such as apoptosis (17), ischemia (37, 38), and chemical toxicity (39, 40). The injury signal produced by the 6-OHDA lesion and infusion cannulae in our study may similarly result in a cascade of timed microenvironmental stimuli that may promote the migration en masse of neural progenitors from the SVZ in response to TGFα. Although other factors may ultimately interact to produce optimal PMD under various experimental and clinical conditions, it is surprising that application of a single neurotrophic factor (TGFα) in an injured brain region is sufficient to effect such significant repair mechanism(s) with positive functional results. TGFα may have several mechanisms of action, perhaps through interactions with multiple TGFα/EGF receptor subtypes (41), which lead to behavioral recovery, such as PMD of stem and progenitor cells, which replace lost circuits and functions; induction of new phenotypic expression in preexisting cells; and neuroprotection against cytotoxic or apoptotic signals. TGFα may be an important endogenous trophic factor in both central and peripheral tissues throughout development, adulthood, and in response to injury and degeneration of tissue. For example, an increase in TGFα levels has been measured in the striata of some Parkinson's disease patients (42, 43). Perhaps the TGFα released endogenously in degenerative disorders is not at a high enough concentration to offset the progressive neural loss with a compensatory stimulation of PMD. Exogenously administered TGFα over a threshold level in patients with Parkinson's disease or other chronic and acute neural damage may then lead to system-specific regeneration and protection of neural circuitry, as well as reversal of clinical symptoms.

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